regulated by Fur was evaluated to assess whether expression of the $Fe^{3+}$-dicitrate transporter contributed to the increase in the SodB activity. The *fecA1* mRNA expression in KS0048 and KS0145 was significantly derepressed compared with that in ATCC700392 and KS0189 under normal cultivation conditions (Fig. 3A). Interestingly, the *fecA2* mRNA expression was not derepressed under normal cultivation conditions (Fig. 3B). These results suggest that the enhanced SodB activities of KS0048 and KS0145 were related to the increase in the $Fe^{3+}$-dicitrate uptake mediated by the *fecA1* gene. Next, to assess the mechanisms of *fecA1* mRNA derepression in KS0048 and KS0145, we aligned the nucleic acid sequences of the Fur-binding consensus sequence (Fur-box: AACTAATAATGGTTATT) of the *fecA1* promoter [15] and then examined the binding affinity of the iron-bound wild-type Fur and iron-bound mutant Fur to the promoters of *fecA1* and *fecA2* by surface plasmon resonance assay (BIAcore2000). No distinct mutation in the *fecA1* promoter was observed in KS0048 and KS0145 (data not shown). The $K_d$ value of the binding of iron-bound mutant Fur to the *fecA1* and *fecA2* promoters as control was measured in comparison with that of iron-bound wild-type Fur. The results of the BIAcore assay revealed a significant increase in the $K_d$ value for binding of iron-bound mutant Fur to the *fecA1* promoter compared with that of iron-bound wild-type Fur to the *fecA1* promoter (Fig. 3C), indicating a significantly reduced affinity of iron-bound mutant Fur for the *fecA1* promoter; therefore, *fecA1* expression was derepressed to a greater extent in KS0048 and KS0145 than in ATCC700392 and KS0189. On the other hand, the $K_d$ value of iron-bound mutant Fur binding to the *fecA2* promoter did not increase (Fig. 3D), indicating that the amino acid mutations in Fur did not influence binding affinity to the *fecA2* promoter.

### *$H_2O_2$ sensitivity and Mtz resistance in the Mtz-resistant strains with mutant Fur under iron-restricted conditions*

Next, we expected that the enhanced SodB activity in KS0048 and KS0145 might be repressed by iron-restricted conditions, to increase the $H_2O_2$ sensitivity and decrease Mtz resistance. First of all, to characterize the $H_2O_2$ sensitivity under iron-restricted conditions, we used an inhibition zone assay to comparatively examine the sensitivity of the ATCC700392, KS0189, KS0048, KS0145, and SodB-overexpressing mutants (ATCC700392 pHel3::sodB). The $H_2O_2$ sensitivity of KS0048, KS0145, and ATCC700392 pHel3::sodB was significantly decreased compared with that of ATCC700392 under normal cultivation conditions (Table 1). Under iron-restricted conditions, on the other hand, whereas the $H_2O_2$ sensitivity of ATCC700392 pHel3::sodB increased to the same level as that of ATCC700392, that of KS0048 and KS0145 was significantly lower compared with that of ATCC700392 (Table 1). Similarly, although the Mtz resistance of ATCC700392 pHel3::sodB (MIC = 32 μg/ml) [6] decreased to the level of Mtz sensitivity (MIC < 8 μg/ml) under iron-restricted conditions (MIC = 4 μg/ml), no decrease in the Mtz resistance of KS0048 and KS0145 was observed (Table 1). A possible reason for this finding is that the SodB activity was significantly higher in KS0048 and KS0145 compared with that in ATCC700392 and KS0189 under iron-

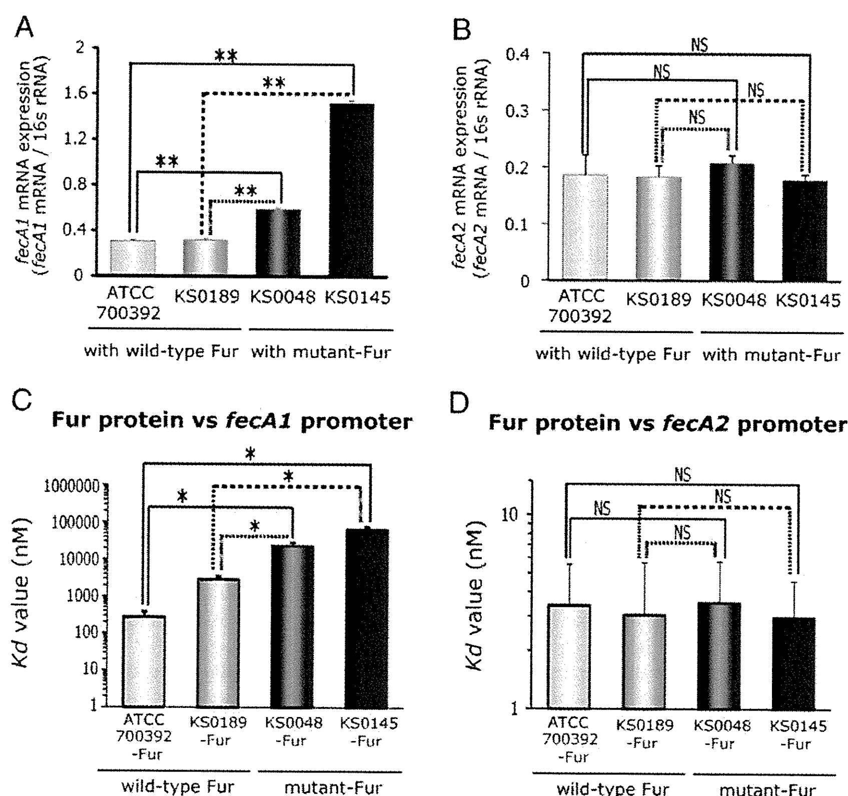


**Fig. 3.** Derepression of *fecA1* mRNA transcription by decreased affinity of mutant Fur for the *fecA1* promoter. (A) Under normal cultivation conditions, transcription of *fecA1* mRNA in ATCC700392 and KS0189, KS0048, and KS0145 was measured by quantitative RT-PCR. Results are means ± SD of three independent assays. $^{**}P<0.01$, statistically significant difference compared with the *fecA1* mRNA expression in ATCC700392 and KS0189. (B) Under normal cultivation conditions, transcription of *fecA2* mRNA in ATCC700392 and KS0189, KS0048, and KS0145 was measured by quantitative RT-PCR. Results are means ± SD of three independent assays. NS, not significant. (C) The $K_d$ value for binding of each Fur protein to the *fecA1* promoter was calculated as a reference in the non-*fecA1* promoter-immobilized flow cells using BIAevaluation software. The white bar indicates the affinity of wild-type Fur for the *fecA1* promoter, and the black bar indicates the affinity of mutant Fur for the *fecA1* promoter. Results are means ± SD of three independent assays. $^{*}P<0.05$, statistically significant difference compared with ATCC700392-Fur and KS0189-Fur. (D) The $K_d$ value for binding of each Fur protein to the *fecA2* promoter was calculated as a reference in the non-*fecA2* promoter-immobilized flow cells using BIAevaluation software. The white bar indicates the affinity of wild-type Fur for the *fecA2* promoter, and the black bar indicates the affinity of mutant Fur for the *fecA2* promoter. Results are means ± SD of three independent assays. NS, not significant.

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**Table 1**
$H_2O_2$ and Mtz resistance of Mtz-resistant strains carrying mutant Fur under iron-restricted conditions.

| Strain | Substitutions in Fur [6] | Iron-replete (normal cultivation) condition | | Iron-restricted condition (20 μM deferoxamine mesylate) | | |
|---|---|---|---|---|---|---|
| | | Mean inhibition zone (mm) | P value | Mean inhibition zone (mm) | P value | MIC (μg/ml) |
| ATCC700392 | Wild type | 2.8±0.29 | | 4.5±0.89 | | <0.5 |
| KS0189 | N118H | 3.4±0.52 | 0.18 | 4.6±0.40 | 0.91 | <0.5 |
| KS0048 | P114S, N118H | 1.9±0.10 | 0.006** | 2.2±0.25 | 0.013* | 16 |
| KS0145 | C78Y, N118H | 1.6±0.47 | 0.020** | 2.1±0.12 | 0.042* | 32 |
| ATCC700392, pHel3::sodB | Wild type | 1.6±0.45 | 0.019** | 4.3±0.58 | 0.78 | 4 |
| ATCC700392, pHel3 control | Wild type | 3.0±0.45 | 0.69 | 4.5±0.30 | 1.0 | 2 |

Results are means ± SD of three independent assays.MIC, minimum inhibitory concentration (μg/mL).
*$P<0.05$ compared with ATCC700392.

restricted conditions (Fig. 4A). From this result, we expected that KS0048 and KS0145 might show enhanced iron ($Fe^{2+}$ and $Fe^{3+}$)-storage ability under normal cultivation conditions and then may make efficient reuse of the ferrous ion under iron-restricted conditions. Therefore, to examine the $Fe^{2+}$-storage ability of KS0048 and KS0145, we evaluated the mRNA expression of ferritin *pfr*, which is the major $Fe^{2+}$-storage protein regulated by Fur in *H. pylori* under normal cultivation conditions [23-25]. The expression levels of *pfr* mRNA in KS0048 and KS0145 were significantly increased compared with those in ATCC700392 and KS0189 under normal cultivation conditions (Fig. 4B). Actually, the levels of intracellular iron ($Fe^{2+}$ and $Fe^{3+}$) in KS0048 and KS0145 were significantly higher than those in ATCC700392 and KS0189 under normal cultivation conditions (Fig. 4C). Additionally, under iron-restricted conditions, the *pfr* mRNA expression in the KS0048 and KS0145 strains was significantly derepressed compared with that in ATCC700392 and KS0189 (Fig. 4D), and the levels of intracellular iron in the KS0048 and KS0145 strains were also increased (Fig. 4E). These results suggested that KS0048 and KS0145 have an enhanced capability for ferrous ion storage by derepression of *pfr* under both normal cultivation and iron-restricted conditions.

### *Contribution of FecA1 to SodB activity, $H_2O_2$ sensitivity, and Mtz resistance*

To characterize the contribution of FecA1 to the SodB activity, $H_2O_2$ sensitivity, and Mtz resistance of *H. pylori*, we constructed a *fecA1*-deletion mutant strain of each *H. pylori* strain (ATCC700392 *fecA1*-deletion mutant, KS0048 *fecA1*-deletion mutant, and KS0145 *fecA1*-deletion mutant). Deletion of the *fecA1* gene hardly influenced the bacterial growth in this study (data not shown). One reason for this may be that there was no decrease in the uptake of Fe ions (both $^{55}Fe^{2+}$ and $^{55}Fe^{3+}$) into the bacterial cells of the *fecA1*-deletion mutant strains [26]. The SodB activity of all *fecA1*-deletion mutant strains was significantly decreased (Fig. 5A). The SodB activity of ATCC700392 was the most significantly decreased with *fecA1* deletion, suggesting that $Fe^{2+}$ is supplied to SodB through FecA1 in *H. pylori*, regardless of the presence/absence of amino acid mutations in Fur. Similarly, the $H_2O_2$ resistance of each *fecA1*-deletion mutant was significantly decreased by 30–60% (Fig. 5B). In addition, the MICs of Mtz for KS0048 and KS0145 decreased dramatically from 32 to 4 and from 128 to 32 μg/ml, respectively. Especially, the Mtz resistance of KS0048 was completely reversed by *fecA1* deletion (MIC<8 μg/ml). To assess whether derepression of *fecA1* mRNA expression was dependent on mutant Fur, we measured the MIC of Mtz in a *fecA1*-deletion mutant of ATCC43504. Development of Mtz resistance in ATCC43504 was caused by the deletion of the *rdxA* gene [18]. Alignment of the predicted amino acid sequences of ATCC43504-Fur showed that ATCC43504-Fur was the wild type. This sequence showed a 100% homology with KS0189-Fur (data not shown). The MIC of Mtz for ATCC43504 decreased slightly (from 128 to 64 μg/ml) after *fecA1* deletion. This finding demonstrated that development of Mtz resistance by FecA1 depended on the mutant Fur.

### *Colonization of Mongolian gerbils by the fecA1-deletion mutant*

To assess the role of FecA1 in the host-colonization ability of *H. pylori*, we measured the colonization of the gastric mucosa by wild-type and *fecA1*-deletion mutant strains at 12 weeks after inoculation into Mongolian gerbils. The *fecA1*-deletion mutant of ATCC700392 tended to show reduced host colonization compared with the wild-type ATCC700392 ($P=0.050$; Fig. 6). The *fecA1*-deletion mutants KS0048 and KS0145 showed a significantly reduced capability for host colonization compared with the wild type of each strain ($P=0.014$ and $P=0.016$, respectively; Fig. 6). Our finding did not indicate whether the host-colonization abilities of KS0048 and KS0145 were significantly increased compared with that of the ATCC700392 (Fig. 6). This result suggested that derepression of *fecA1* by mutant Fur alone did not lead to enhanced host colonization.

## Discussion

*H. pylori* encodes only one single iron ($Fe^{2+}$)-cofactored SOD (SodB). Therefore, ferrous ion is indispensable for activation of SOD in *H. pylori* [8]. Our findings indicate that the enhanced $Fe^{2+}$-supply system associated with SodB activation in the KS0048 and KS0145 strains can be explained as follows: under iron-replete conditions, $Fe^{3+}$-dicitrate transport was enhanced by derepression of *fecA1* mRNA expression by iron-bound mutant Fur. Intracellular ferric ion ($Fe^{3+}$) was reduced to $Fe^{2+}$ by $Fe^{3+}$-reductase (ribBA) [27], providing $Fe^{2+}$ to SodB (Fig. 7). Under iron-restricted conditions, $Fe^{2+}$ storage in KS0048 and KS0145 was enhanced through derepression of *pfr* mRNA expression by apo-mutant Fur, supplying $Fe^{2+}$ to SodB (Fig. 7). In addition, our results demonstrated, for the first time, that FecA1 may play an indispensable role in the bacterial survival in the stomach and in the development of Mtz resistance of *H. pylori* through $Fe^{2+}$ supply to SodB.

Because *H. pylori* is a highly genetically diverse organism, different strains may show great variations in phenotype. However, in this study, all *fecA1*-deletion mutant strains of *H. pylori* (ATCC700392, KS0048, and KS0145) showed reduced SodB activity and reduced gastric mucosal colonization ability. Therefore, it is thought that $Fe^{3+}$-dicitrate transport by FecA1 is associated with the activation of SodB, regardless of the genetic diversity of the strains.

The *fecA1* and *fecA2* genes, encoded in *H. pylori* as a $Fe^{3+}$-dicitrate transporter, are both regulated by Fur [10,11]. In this study, interestingly, in KS0048 and KS0145, only *fecA1* expression was derepressed by mutant Fur under normal cultivation conditions (Fig. 3A), whereas the expression of *fecA2* was repressed (Fig. 3B). The underlying reason was the high affinity of iron-bound wild-type Fur for the *fecA2* promoter compared with that for the *fecA1* promoter; the $K_d$ value of iron-bound wild-type Fur binding to the *fecA2* promoter ($K_d=3.4$ nM) was low compared with that to the *fecA1* promoter ($K_d=273$ nM) (Figs. 3C and D). This result suggested that the expression of *fecA1*, but not of *fecA2*, was more influenced by amino acid mutations in Fur, and then only the expression of *fecA1* was derepressed by mutant Fur. Recently, Ernst et al. [5] reported that the $K_d$ value for the *sodB* promoter of apo-wild-type Fur was also low ($K_d=270$ nM, from the data of [5]), similar to the $K_d$ value for the *fecA1* promoter of iron-bound wild-type Fur. Because *H. pylori* is continuously exposed to superoxides generated by its own respiration and metabolism and the

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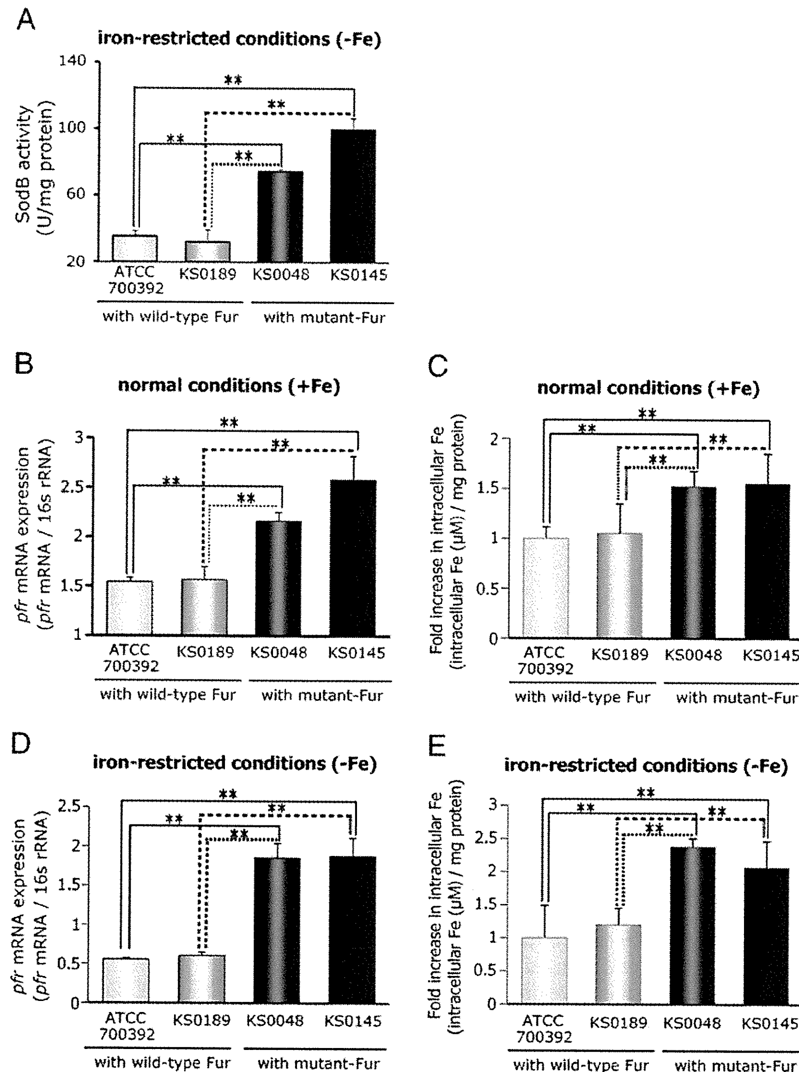


**Fig. 4.** SodB activity of the Mtz-resistant strains with mutant Fur under iron-restricted conditions was supported by the increase in the iron-storage ability. (A) Under iron-restricted conditions, the SodB activity in ATCC700392, KS0189, KS0048, and KS0145 was measured by the method described under Materials and methods. Results are means ± SD of three independent assays. $^{**}P<0.01$, statistically significant difference compared with the SodB activity in ATCC700392 and KS0189. (B) Under normal cultivation conditions, expression of *pfr* mRNA in ATCC700392, KS0189, KS0048, and KS0145 was measured by quantitative RT-PCR. Results are means ± SD of three independent assays. $^{**}P<0.01$, statistically significant difference compared with *pfr* mRNA expression in ATCC700392 and KS0189. (C) Under normal cultivation conditions, intracellular iron ($Fe^{2+}$ and $Fe^{3+}$) concentration was measured by the method described under Materials and methods. The data for the intracellular iron concentration were corrected by total cellular protein. Results are means ± SD of three independent assays. $^{**}P<0.01$, statistically significant difference compared with the intracellular iron concentration in ATCC700392 and KS0189. (D) Under iron-restricted conditions, expression of *pfr* mRNA in ATCC700392, KS0189, KS0048, and KS0145 was measured by quantitative RT-PCR. Results are means ± SD of three independent assays. $^{**}P<0.01$, statistically significant difference compared with *pfr* mRNA expression in ATCC700392 and KS0189. (E) Under iron-restricted conditions, intracellular iron ($Fe^{2+}$ and $Fe^{3+}$) concentration was measured by the method described under Materials and methods. Results are means ± SD of three independent assays. $^{**}P<0.01$, statistically significant difference compared with the intracellular iron concentration in ATCC700392 and KS0189.

host immune response, sustained expression of SodB activity is required for the dismutation of such superoxides [28,29]. Hence, it is thought that a low affinity of apo-Fur and iron-bound Fur for the *sodB* and *fecA1* promoters, respectively, is required for efficient and persistent activation of SodB.

In *H. pylori*, Fur regulates the gene expression of both iron-bound and apo-Fur [11]. *sodB* mRNA expression is repressed by apo-Fur; on the other hand, *fecA1* mRNA expression is repressed by iron-bound Fur [5,14,15]. In fact, despite the difference in the binding patterns of Fur to the *sodB* promoter and *fecA1* promoter, the mRNA expression of both *sodB* and *fecA1* was co-derepressed by mutant Fur (Fig. 3) [6], suggesting that the amino acid mutations (C78Y and P114S) in Fur alter its binding to promoter DNA, but not to $Fe^{2+}$. *H. pylori* Fur monomer contains two domains, the N-terminal DNA-binding domain and the C-terminal dimerization domain with metal-binding sites, and after dimerization, the Fur protein binds to the target promoter DNA [6,30]. We showed, using homology modeling, that the mutation C78Y was localized in the DNA-binding domain, whereas P114S was localized in the oligomerization domain [6]. Changes in the target-DNA binding of Fur by amino acid mutation have been categorized into the following two groups: (i) effects on the binding ability of $Fe^{2+}$ and (ii) effects on dimerization [30]. Dian et al. identified the S2 functional domain, which was essential for dimerization in *H. pylori* Fur [31]. According to that report, replacement of Cys 78 with tyrosine is predicted to interfere with the formation of the S2 site [31].

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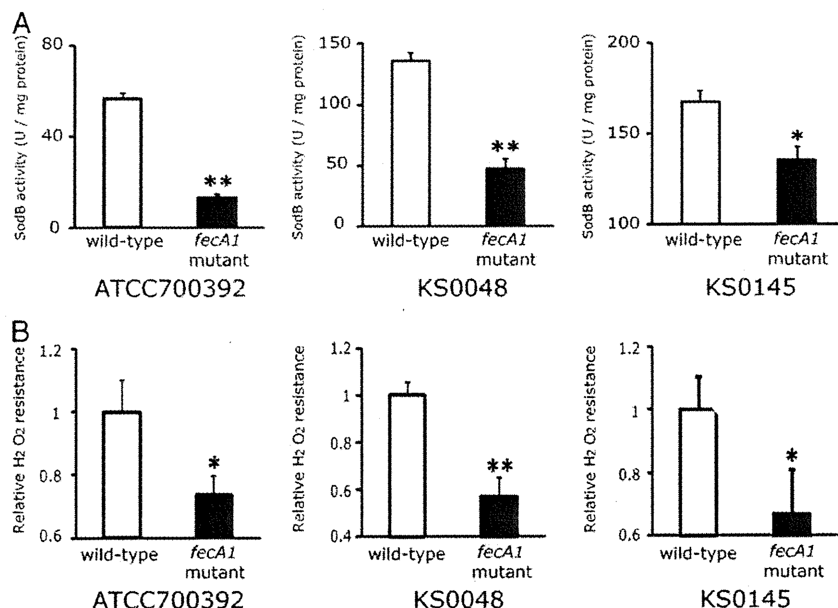


**Fig. 5.** Deletion of *fecA1* reduces SodB activity and $H_2O_2$ resistance. (A) The *fecA1*-deletion mutant was constructed as described under Materials and methods. The SodB activity in the wild-type and *fecA1*-deletion mutants for each strain was measured as described under Materials and methods. The results are expressed as means ± SD of three independent assays. *$P<0.05$, **$P<0.01$, statistically significant difference compared with the wild-type for each strain. (B) $H_2O_2$ resistance was measured by the inhibition zone assay described under Materials and methods. The results are means ± SD of three independent assays. *$P<0.05$, **$P<0.01$, statistically significant difference compared with the wild-type for each strain.

Our in vivo studies demonstrated that the colonization ability of *H. pylori* in Mongolian gerbils was greatly impaired by *fecA1* deletion, regardless of the presence of Fur mutation. From this result, it is thought that the SodB activation in *H. pylori* is supported by $Fe^{2+}$ supply through FecA1 to combat the oxidative stress evoked by the host immune response. Because recently there has been a gradual increase in reports of multiple-drug-resistant *H. pylori*, the development of a novel bactericidal therapy, different from antibiotics, is required. FecA1 is one possible target for the development of a novel bactericidal therapy as well as possibly a preventive therapy against *H. pylori* infection.

In conclusion, $Fe^{3+}$-dicitrate transport by FecA1 is an essential process in the activation of SodB, which determines the gastric mucosal colonization ability of *H. pylori* in Mongolian gerbils and also the development of Mtz resistance.

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.freeradbiomed.2011.12.011.

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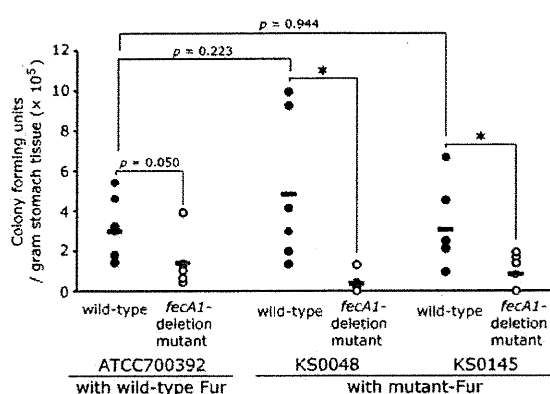


**Fig. 6.** Deletion of *fecA1* reduces the ability of *H. pylori* to colonize the stomach of Mongolian gerbils. Total colonization of the stomach was determined by sacrificing the animals at 12 weeks, and the results were expressed as the number of CFU/g of stomach tissue. Mongolian gerbils were infected with either a wild-type *H. pylori* strain (filled circle) or a *fecA1*-deletion mutant *H. pylori* strain (open circle). Each circle indicates the results for a single animal. The geometric means are indicated by bars. *$P<0.05$, statistically significant difference compared with the wild type.

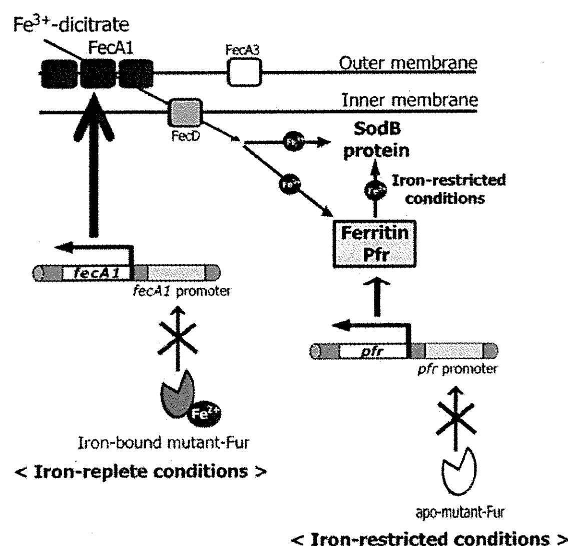


**Fig. 7.** Schematic representation of the ferrous ion ($Fe^{2+}$)-supply system to the SodB protein in the Mtz-resistant strains with mutant Fur. Under iron-replete conditions, *fecA1* mRNA expression is derepressed by iron-bound mutant Fur, and then $Fe^{2+}$ is supplied to the SodB protein. Under iron-restricted conditions, the capability of $Fe^{2+}$ storage in the KS0048 and KS0145 strains is enhanced by derepression of *pfr* mRNA expression by apo-mutant Fur, and then $Fe^{2+}$ is supplied to SodB from Pfr.

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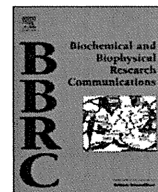

# Effects of β-(1,3–1,6)-D-glucan on irritable bowel syndrome-related colonic hypersensitivity

Teita Asano [a], Ken-ichiro Tanaka [b], Shintaro Suemasu [a], Tomoaki Ishihara [a], Kayoko Tahara [a], Toshio Suzuki [c], Hidekazu Suzuki [d], Shin Fukudo [e], Tohru Mizushima [a,b,*]

[a] Department of Analytical Chemistry, Faculty of Pharmacy, Keio University, Tokyo 105-8512, Japan
[b] Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto 862-0973, Japan
[c] Research and Development, Daiso Co., Ltd., Amagasaki 660-0842, Japan
[d] Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo 160-8582, Japan
[e] Department of Behavioral Medicine, Tohoku University Graduate School of Medicine, Sendai 980-8575, Japan

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ABSTRACT

Irritable bowel syndrome (IBS) is a gastrointestinal disorder characterized by chronic abdominal pain associated with altered bowel habits. Since the prevalence of IBS is very high and thus, involves elevated health-care costs, treatment of this condition by methods other than prescribed medicines could be beneficial. β-(1,3)-D-glucan with β-(1,6) branches (β-glucan) has been used as a nutritional supplement for many years. In this study, we examined the effect of β-glucan on fecal pellet output and visceral pain response in animal models of IBS. Oral administration of β-glucan suppressed the restraint stress- or drug-induced fecal pellet output. β-Glucan also suppressed the visceral pain response to colorectal distension. These results suggest that β-glucan could be beneficial for the treatment and prevention of IBS.
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## 1. Introduction

IBS is a functional gastrointestinal disorder characterized by chronic and recurrent abdominal pain and discomfort (colonic hypersensitivity) that are associated with altered bowel habits but not with any detectable structural or biochemical abnormality [1,2]. IBS is categorized into subtypes according to the predominant bowel habit: diarrhea-predominant IBS, constipation -predominant IBS, and mixed pattern IBS [1]. In spite of the significant impact that IBS has on patient quality-of-life, currently available clinical treatments for IBS have proved unsatisfactory, mainly due to the difficulty in suppressing the visceral pain associated with IBS.

IBS is one of the most common gastrointestinal disorders, estimated to affect 7–15% of the general population in the USA and 6–12% in Asian countries [2,3]. Considering the health-care costs associated with treating the condition, the identification of effective therapies (such as the taking of supplements) that do not involve prescription drugs is beneficial [4,5].

Although the pathogenesis of IBS is not completely understood, studies have suggested that genetic factors, previous inflammation, mental stressors and microbiota play important roles [6]. A number of animal models for IBS has been established and used to evaluate clinical protocols designed to treat the condition. Mental stressor- or drug-induced alterations in defecation have been used as a model for defecation disorders related to IBS in animals [7–9]. Since hypersensitivity to colorectal distension (CRD) was observed in IBS patients [10], monitoring the electrical activity of the abdominal muscles (visceromotor response) in response to CRD is a standard procedure to detect IBS-related abdominal pain (visceral pain) in animals [11,12]. Furthermore, based on the increased colonic level of butyrate in IBS patients [13,14], butyrate enema-induced hypersensitivity to CRD is also considered as a useful animal model for IBS [15,16].

β-Glucans are naturally-occurring polysaccharides found in the cell walls of yeast, fungi, cereal plants and certain bacteria [17,18]. As suggested by the fact that various foods contain β-glucans, they are known to have few toxic and adverse effects [18]. β-Glucans from mushrooms have been used in Japan as anti-tumor drugs due to their immunostimulating activities [17]. In addition, β-(1,3)-D-glucans with β-(1,6) branches have been reported to have various clinically beneficial effects, such as enhancing the

*Abbreviations:* AUC, area under the curve; β-glucan, β-(1,3)-D-glucan with β-(1,6) branches; CRD, colorectal distention; 5-HT, 5-hydroxytryptamine hydrochloride; IBS, irritable bowel syndrome; LMW, low-molecular-weight; PBS, phosphate-buffered saline; S.E.M, standard error of the mean.

* Corresponding author at: Department of Analytical Chemistry, Faculty of Pharmacy, Keio University, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512, Japan. Fax: +81 3 5400 2628.
*E-mail address:* mizushima-th@pha.keio.ac.jp (T. Mizushima).

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bio-defense activity against bacterial, viral, fungal and parasitic challenge, increasing hematopoiesis and radioprotection, stimulating the wound healing response, and decreasing serum lipid levels [17–20]. Interestingly, it was recently reported that β-glucans suppress inflammatory responses in some animal models [21–26], suggesting that β-glucan could be an interesting immunomodulator, causing opposing effects on different aspects of the immune system.

We succeeded in the purification and industrial-scale production of low-molecular-weight β-(1,3–1,6)-D-glucan from *Aureobasidium pullulans* (*A. pullulans*) GM-NH-1A1 strain (LMW β-glucan) [27,28]. The characteristic features of LMW β-glucan are its low molecular weight (about 100 kDa), low viscosity, high water-solubility and high level of β-(1–6) branching (50–80%) [27,28]. We previously reported that LMW β-glucan has various clinically beneficial effects, such as suppression of the allergic response, suppression of restraint stress-induced immunosuppression and anti-tumor and anti-metastatic actions [27–29]. Moreover, we recently reported that LMW β-glucan protects the gastric mucosa against the formation of irritant-induced lesions by increasing levels of defensive factors such as heat shock protein 70 and gastric mucin [30]. In the present study, we use different animal models for IBS to test the hypothesis that LMW β-glucan could be effective in the treatment of this condition. Our results suggest that the oral administration of LMW β-glucan suppresses not only fecal pellet output but also the visceromotor response to CRD (visceral pain response). These findings suggest that LMW β-glucan could be therapeutically effective for the treatment of IBS.

## 2. Materials and methods

### 2.1. Chemicals and animals

LMW β-glucan was prepared from the conditioned culture medium of *A. pullulans* GM-NH-1A1, as described previously [27,28]. Analysis of $^{1}H$ and $^{13}C$ NMR spectra and gel-filtration chromatography revealed that the LMW β-glucan contains approximately 70% β-(1–6) branches and an average molecular weight of 100 kDa, as described previously [27,28]. Clonidine hydrochloride and castor oil were from WAKO Pure Chemicals (Osaka, Japan). Sodium butyrate, brewer's yeast and carbamyl- β-methylcholine chloride (bethanecol) were obtained from Sigma (St. Louis, MO). Loperamide hydrochloride and 5-hydroxytryptamine hydrochloride (5-HT) were purchased from Nacalai Tesque (Kyoto, Japan). Wild-type mice (C57/BL6, 6–8 weeks of age) and Wistar rats (4–6 weeks of age) were obtained from Charles River (Yokohama, Japan). Wistar-Imamichi rats (4 weeks of age) were purchased from the Institute for Animal Reproduction (Kasumigaura, Japan). The experiments and procedures described here were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health, and were approved by the Animal Care Committees of Keio University and Kumamoto University.

### 2.2. Analysis of fecal pellet output in mice

Female mice were subjected to restraint stress by being placed individually into a 50 ml Falcon tube (Becton Dickinson, Franklin Lakes, NJ) for 1 h, as described previously [31]. These tubes are small enough to restrain a mouse so that it is able to breathe but unable to move freely. Control mice were left to move freely in the cage. The number of fecal pellets excreted during the 1-h restraint stress period was measured. β-Glucan was dissolved in phosphate-buffered saline (PBS) and administered orally 2 h before animals were subjected to the restraint stress. Control animals were administered PBS.

In a separate experiment, mice were administered one of different drugs that stimulate intestinal motility (bethanecol and 5-HT), cause diarrhea (castor oil) or cause constipation (loperamide and clonidine). Animals were then placed in a cage and the number or wet weight of fecal pellets excreted in the subsequent 1-, 2- or 24-h period determined. Drugs administered subcutaneously were bethanechol (3 mg/kg) and 5-HT (3 mg/kg), while those administered orally were loperamide (10 mg/kg), clonidine (3.5 mg/kg) and castor oil (300 μl/mouse).

β-Glucan was dissolved in PBS and administered orally 2 h before animals were subjected to the restraint stress or drug-treatment. Control animals were administered PBS.

### 2.3. Electromyography and CRD

Rats were deeply anaesthetized with pentobarbital sodium (40 mg/kg) and then electromyography electrodes (Star Medical, Tokyo, Japan) sutured into the external oblique muscle of the abdomen for electromyogram recording. Electrode leads were tunneled subcutaneously and exteriorized at the nape of the neck for future access. After surgery, rats were housed individually and allowed to recuperate for 6 days before being used for visceromotor response testing.

Repeated CRD was performed as described previously [32]. Rats were restrained in a plastic conical-shape tube (diameter, 6 cm; height, 15 cm), 15 min before electromyography. To reduce confounding effects due to restraint stress, rats were habituated to the tube 30 min per day for 3 days prior to the experiment. A polyethylene bag (length 2 cm) was inserted in the distal colon, positioned 1 cm proximal to rectum, and connected to a balloon catheter which was anchored with tape to the base of the tail. The pressure and volume of the balloon were controlled and monitored by a pressure controller-timing device (Distender Series II; G & J Electronics, Toronto, Canada), connected to the balloon. Rats were subjected to repeated CRD (80 mm Hg, 30 s, 5-min interstimulus interval, 12 times) on day 7. β-Glucan was given orally once daily for 7 days (from day 0 to day 6).

In separate experiments, CRD associated with the use of butyrate enemas was examined as described previously [15]. Rats were instilled with 1 ml sodium butyrate (110 mg/ml, pH 6.9) or saline into the colon twice daily for 3 days (day 1, 2 and 3). Rats were subjected to CRD (10, 20, 40 60 and 80 mm Hg, 20 s, 150-s interstimulus interval) on day 7. β-Glucan was given orally once daily for 7 days (from day 0 to day 6).

Visceromotor responses were monitored by electromyography, as described previously [11,33], 12 h after the last administration of β-glucan. Electromyograph data were collected and analyzed using 8 STAR software (version 6.0–19.2 for Windows; Star Medical, Tokyo, Japan). Responses evoked by contraction of the external oblique musculature were quantified by calculating the area under the curve (AUC) of the voltage alteration graph. The baseline was determined by data collected 20 s (butyrate enema) or 30 s (repeated CRD) before each distention.

### 2.4. Inflamed paw pressure nociception test

The pain threshold in Wistar-Imamichi rats was measured using a Randall–Sellito test with an analgometer (Ugo basile, Comerio, Italy), as described previously [34]. Brewer's yeast (20%, 1 ml) was injected into one of the hind paws. Seven hours later, an increasing pressure was applied to the underside of the hind limb and the pain threshold was defined as the pressure in grams eliciting a cry from the animal.

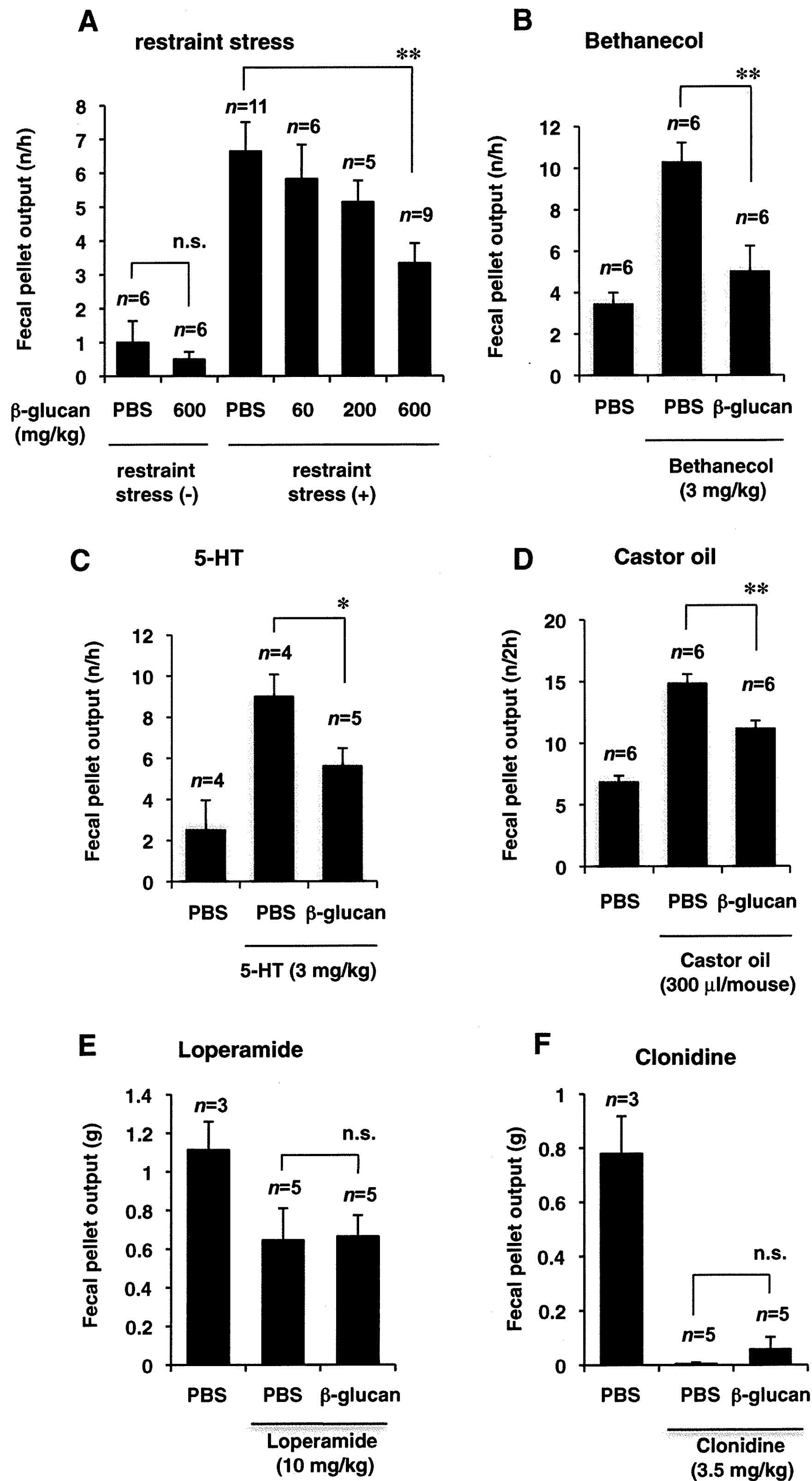


**Fig. 1.** Effects of LMW β-glucan on restraint stress- or drug-induced alteration of fecal pellet output in mice. Mice were orally administered indicated doses (A) or 600 mg/kg (B–F) of LMW β-glucan or vehicle (PBS). Two hours later, mice were exposed to restraint stress (A) or administered bethanecol (3 mg/kg, *s.c.*) (B), 5-HT (3 mg/kg, *s.c.*) (C), castor oil (300 μl/mouse, *p.o.*) (D), loperamide (10 mg/kg, *p.o.*) or clonidine (3.5 mg/kg, *p.o.*). The number (A–D) or wet weight (E and F) of fecal pellets excreted in the subsequent 0–1 h (A–C), 0–2 h (D) or 0–24 h (E and F) period was determined. Values are mean ± S.E.M $^{*}P < 0.05$; $^{**}P < 0.01$; n.s., not significant.

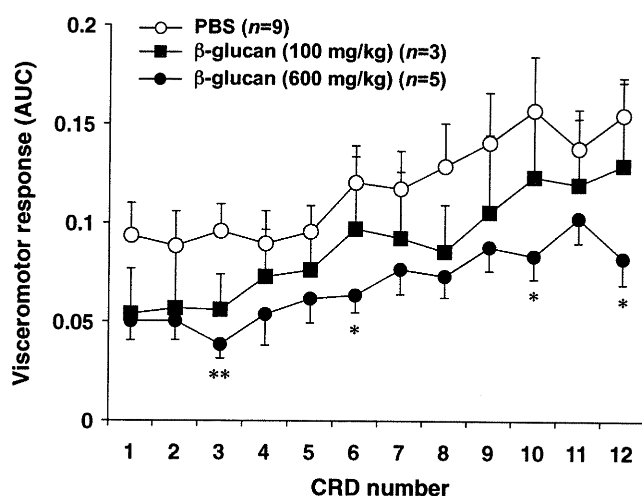


**Fig. 2.** Effect of LMW β-glucan on the viscermotor response to CRD in rats. The indicated doses (mg/kg) of β-glucan or PBS were orally administered to female Wistar rats once daily for 7 days. Twelve hours after the last administration of LMW β-glucan, rats were subjected to repetitive CRD and the visceromotor response was recorded and analysed as described in Section 2. Values are mean ± S.E.M $^*P < 0.05$; $^{**}P < 0.01$.

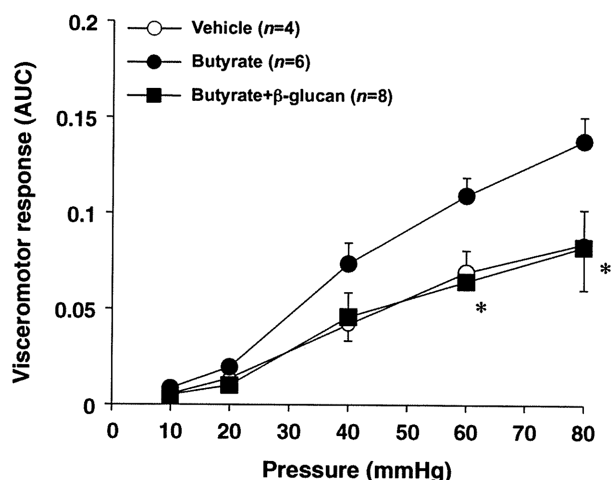


**Fig. 3.** Effect of LMW β-glucan on butyrate enema-induced colonic hypersensitivity to CRD in rats. Butyrate enemas were administered twice daily on days 1, 2 and 3. Administration of LMW β-glucan (600 mg/kg) (once daily from day 0 to day 6) and monitoring and analysis of the visceromotor response to CRD (on day 7) were performed as described in the legend of Fig. 2. Values are mean ± S.E.M $^*P < 0.05$.

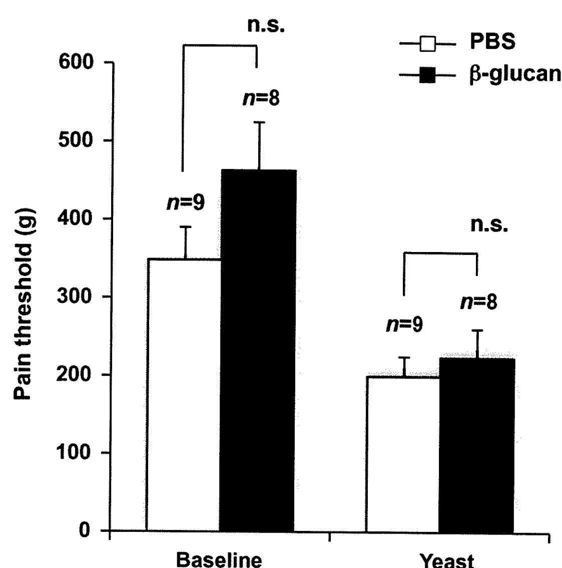


**Fig. 4.** Effect of LMW β-glucan on the pain response of rats in the inflamed paw pressure test. LMW β-glucan (600 mg/kg) was administered orally as described in the legend of Fig. 2. Twelve hours after the last administration of β-glucan, the inflamed paw pressure test was performed as described in Section 2. The pain threshold before (baseline) and after (yeast) the yeast injection was determined. Values are mean ± S.E.M n.s., not significant.

*2.5. Statistical analysis*

All values are expressed as the mean ± S.E.M. Two-way ANOVA followed by the Tukey test or a Student's *t* test for unpaired results was used to evaluate differences between more than two groups or between two groups, respectively. Differences were considered to be significant for values of $P < 0.05$.

## 3. Results and discussion

*3.1. Effect of LMW β-glucan on fecal pellet output in mice*

We first examined the effect of a once-only oral administration of LMW β-glucan on restraint stress-induced fecal pellet output in mice. In untreated mice (administered PBS vehicle only), restraint stress (restricted movement by placement of mouse in a 50 ml plastic tube) caused a more than 5-fold increase in fecal pellet output per hour compared to unrestrained mice (Fig. 1A), as described previously [35]. The once-only oral pre-administration of LMW β-glucan suppressed this increase in a dose-dependent manner without affecting the basal level (without restraint stress) of fecal pellet output (Fig. 1A). Similar results were observed in response to a once-daily oral administration of LMW β-glucan for 7 days (data not shown). The LMW β-glucan-dependent suppression of restraint stress-induced fecal pellet output was also confirmed in rats (data not shown).

We also examined the effect of LMW β-glucan on the fecal pellet output induced by drugs that increase intestinal motility (bethanecol and 5-HT) or cause diarrhea (castor oil) [8,36]. As shown in Fig. 1B–D, the oral administration of LMW β-glucan (600 mg/kg) to mice suppressed the fecal pellet output induced by each of these drugs.

We then examined the effect of LMW β-glucan on drug-induced constipation. As shown in Fig. 1E and F, administration of loperamide or clonidine to mice decreased fecal pellet output, as described previously [36]. The oral pre-administration of LMW β-glucan did not alter the fecal pellet output. The results in Fig. 1 thus suggest that orally administered LMW β-glucan suppresses the restraint stress- or drug-induced stimulation of intestinal motility but does not affect the motility in the absence of these stimuli or in presence of constipation-inducing drugs. The mechanism underlying the LMW β-glucan-dependent suppression of intestinal motility is not clear at present.

*3.2. Effect of LMW β-glucan on the visceromotor response to CRD in rats*

In addition to alterations of fecal pellet output, hypersensitivity to visceral pain is one of the principle pathogenetic pathways for IBS. To study this phenomenon, we examined the effect of LMW β-glucan on visceromotor response to CRD, which has been used as an index of visceral pain response [33]. Rats were used for this analysis since the techniques for measuring the visceromotor response and CRD were established with these animals. As a single oral administration of LMW β-glucan did not significantly affect the visceromotor response to CRD (data not shown), we decided

to determine the effect of LMW β-glucan administered orally once-daily for 7 days. In control rats (PBS-treated), CRD evoked a visceromotor response which increased in amplitude in response to repeated CRDs (Fig. 2), as described previously [32]. Oral pre-administration of LMW β-glucan (600 mg/kg) to animals significantly decreased the visceromotor response to CRD not only after repetitive CRDs but also upon the first CRD (Fig. 2). Pre-administration of LMW β-glucan (100 mg/kg) also showed a tendency to decrease the visceromotor response to CRD, however the effect was not statistically significant (Fig. 2). These results indicate that oral pre-administration of high dose of LMW β-glucan suppresses the visceral pain response to CRD.

Since the visceromotor response to the first CRD was reduced by the pre-administration of LMW β-glucan, the results in Fig. 2 can be interpreted to indicate that LMW β-glucan suppresses the visceral pain response to CRD itself, but does not affect the repeated CRD-induced hypersensitivity to visceral pain. However, although we tried to habituate rats to the tube used for CRD experiment (see Section 2), it is possible that the animals entered into a state of restraint-like stress. Thus, it is also possible that LMW β-glucan suppresses the restraint stress-induced hypersensitivity to visceral pain.

We then examined the effect of LMW β-glucan on the visceral pain response in another animal model, butyrate-induced hypersensitivity to CRD. The butyrate enema is known to reduce the threshold of the visceromotor response to CRD [15,16]. We confirmed that twice-daily butyrate enemas (on days 1, 2 and 3) stimulated the visceromotor response to CRD on day 7 and found that when LMW β-glucan was orally pre-administered once daily from day 0 to day 6, the visceromotor response to CRD was similar to that measured in control rats (not given butyrate enemas) (Fig. 3). This result suggests that LMW β-glucan suppresses butyrate-induced hypersensitivity to CRD.

Finally, we tested whether the inhibitory effect of LMW β-glucan on the pain response is specific for visceral pain. For this purpose, we used the inflamed paw pressure test in which a yeast solution was administered to one of hind paws of rats to induce inflammation and the pressure-induced pain response was subsequently determined. As shown in Fig. 4, oral administration of LMW β-glucan once daily for 7 days did not affect the paw pressure required to elicit a nociception response (pain threshold) in both presence and absence of yeast injection. This finding suggests that LMW β-glucan does not affect the pain response in general but specifically affects the visceral pain response.

In conclusion, we have shown here that the oral administration of LMW β-glucan suppresses not only restraint stress- or drug-induced fecal pellet output, but also suppresses the visceral pain response. The difficulty associated with therapeutic management of IBS can be attributed to the fact that both abdominal pain and bowel habit disorders must be addressed. The results presented in this study thus suggest that LMW β-glucan could prove therapeutically beneficial for the prevention and treatment of IBS, especially in relation to the diarrhea-predominant IBS.

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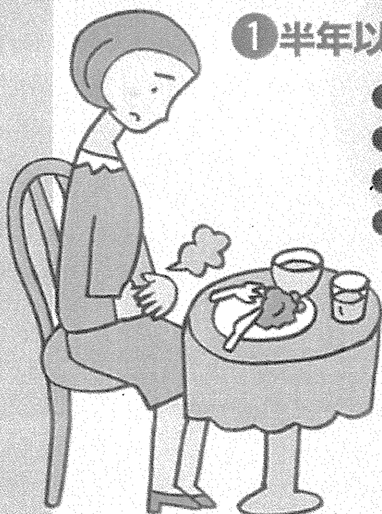
# 胃の調子が悪いとお悩みの方へ

ぜひ、漢方薬の臨床試験にご参加ください

## 今回の臨床試験にご参加いただけるのは次の❶〜❸すべてにあてはまる20歳以上の方です



### ❶半年以上前から次の１つ以上の症状がある

- すぐに食べられなくなってしまう
- 食後に胃がもたれる
- みぞおちに差し込むような痛みがある
- みぞおちが焼けるように感じる

### ❷最近3ヶ月間、次のいずれかの症状がある

- 週に数回、食後の胃もたれや早期飽満感が起きる
- 週に1回以上みぞおちの痛みや焼ける感じがある

※ずっと症状が続いていなくても可

### ❸胃カメラなど胃の検査をしても「異常なし」といわれた

**ご注意** 右記の方は対象外となります

- 未成年の方
- 逆流性食道炎の治療をされている方
- 過去6ヶ月以内にピロリ菌の除菌治療を受けた方
- 妊婦（妊娠している可能性のある）の方
- 胃、食道などの手術をされた方

※上記の他にも参加基準があり、ご参加いただけない場合もあります

詳しい内容はWebサイトでもご覧いただけます http://keio-clinical.jp/

厚生労働科学研究費補助金（医療技術実用化総合研究事業）機能性ディスペプシアに対する六君子湯の有効性・安全性の科学的エビデンスを創出するための多施設共同二重盲検無作為化プラセボ対照比較試験事務局

TEL 070-6946-1119（イイイグスリ） 【受付時間】10:00〜17:00（土・日・祝は除く）
※電話が混み合ってつながりにくい場合もあります。あらかじめご了承ください。

関心のある方は、お気軽にご相談ください

# 胃の調子が悪いとお悩みの方へ

ぜひ、漢方薬の臨床試験にご参加ください

**こういう症状に心当たりのある方は、ぜひ裏面のチェックシートをお試しください**

「内視鏡検査をしても異常がないのに胃の調子が悪い」
かつて「慢性胃炎」や「神経性胃炎」と呼ばれていた病気が、今では「機能性ディスペプシア」という病気として治療を行うようになっています。
今回の臨床試験は、胃の動きを改善することが示唆されている「六君子湯（りっくんしとう）」という漢方薬がこの「機能性ディスペプシア」にどの程度有効なのか、その効果を調べるために行うものです。
具体的には、この「六君子湯」を飲んでいただくグループと偽薬（見た目は「六君子湯」と同じですが薬ではありません）を飲んでいただくグループに分けて、その違いをくらべていきます。

厚生労働科学研究費補助金（医療技術実用化総合研究事業）
機能性ディスペプシアに対する六君子湯の有効性・安全性の科学的エビデンスを創出するための
多施設共同二重盲検無作為化プラセボ対照比較試験

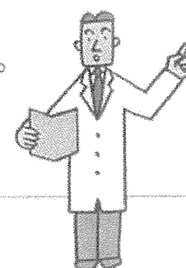
研究代表者：慶應義塾大学医学部内科学（消化器）准教授　鈴木秀和

## チェックシート

今回の臨床試験に参加していただけるかどうか、お確かめください

| | | YES | NO |
|---|---|---|---|
| ① | 20歳以上である。 | | |
| ② | この6ヶ月以内に次の4つのうち1つ以上の症状があった。<br>・食後、胃がもたれてつらい（つらいと感じる食後のもたれ感）<br>・食べはじめても、すぐに食べられなくなってしまう（早期飽満感）<br>・みぞおちに差し込むような痛みがある（心窩部痛）<br>・みぞおちが焼けるように感じる（心窩部灼熱感） | | |
| ③ | この3ヶ月以内にも同じような症状が繰り返しあった。 | | |
| ④ | 暴飲・暴食や風邪薬、痛み止めの薬（ボルタレン、ロキソニン、バファリンなど）を服用したり、急激なストレス等があったりと、症状の原因として思いあたることがある。 | | |
| ⑤ | 風邪薬や痛み止めの薬（ボルタレン、ロキソニン、バファリンなど）、または低用量アスピリン（小児用バファリンやバイアスピリン）を3ヵ月以上服用した際に、上記の胃の症状が出たことがある。 | | |
| ⑥ | 6か月以内に胃カメラかバリウム検査をしたが「異常なし」と診断された。 | | |
| ⑦ | 逆流性食道炎・びらん性胃炎と診断、治療されたことがある。 | | |
| ⑧ | 胃や食道などの手術の経験がある。 | | |
| ⑨ | 脳こうそくなどの脳の病気や、統合失調症またはうつ病と診断されたことがある。 | | |
| ⑩ | アルコール依存または薬物依存と診断されたことがある。 | | |
| ⑪ | 甲状腺機能亢進症などの重いホルモン異常と診断されたことがある。 | | |
| ⑫ | 重い心臓病、肝臓病、腎臓病、感染症または血液病と診断されたことがある。 | | |
| ⑬ | 今回の臨床試験で使用する試験薬剤（六君子湯）の成分に対して過敏症の症状が出たことがある。 | | |
| ⑭ | 妊婦、授乳婦または妊娠している可能性、試験期間中の妊娠の希望などがある。 | | |
| ⑮ | この6ヵ月以内にピロリ菌の除菌治療を受けたことがある。 | | |
| ⑯ | 胃酸を抑える薬、胃腸の動きをよくする薬、胃の動きを止める抗コリン薬、精神安定剤やうつ病の薬などを服薬している。 | | |
| ⑰ | 過敏性腸症候群と診断されて治療している。 | | |

上記の項目で、すべて□欄に当てはまる場合は、
今回の臨床試験に参加していただける可能性がとても高い方です。
ぜひとも下記までご連絡いただくか、
Webサイトで最寄りの医療機関をお確かめのうえ、
お問い合わせください。※上記以外にも参加基準があり、ご参加いただけない場合もあります。



### 参加期間について

・ご参加いただく期間は8週間です。（研究実施期間は2013年1月までです）
・ご自宅で定期的に薬を服用して週1回症状を記入していただきます。
・通院していただくのは、投与前・投与4週間後・8週間後の3回です。（検査には採血があります）

この臨床試験に参加するかしないかは、患者様ご本人の意思が最優先されます。担当医からお話を聞いたあとで、あるいは一度同意されたあとで参加を取りやめても不利益になることは一切ありません。関心を持っていただいた方はお気軽にご相談ください。

お申込み・お問い合わせはこちらまで
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URL http://keio-clinical.jp/ E-Mail dyspepsia@keio-clinical.jp

胃が不快なのに…検査しても異常なし。

# それって機能性ディスペプシア?

検査をしても異常がないのに、胃に不快な症状が続く状態を
「機能性ディスペプシア」といいます。
むずかしそうな名前ですが、
日本人の4人に1人が発症している一般的な病気です。
あなたのその症状、「機能性ディスペプシア」かもしれませんよ。

監修(代表) 慶應義塾大学医学部内科学(消化器)准教授 鈴木 秀和

# こんな症状があったら
# 機能性ディスペプシア

## ❶半年以上前から次の1つ以上の症状がある

すぐに食べられなくなってしまう

食後に胃がもたれる

みぞおちに差し込むような痛みがある

みぞおちが焼けるように感じる

## ❷最近3ヶ月間、次のいずれかの症状がある

週に数回
食後の胃もたれや
早期飽満感が起きる

週に1回以上
みぞおちの痛みや
焼ける感じがある

※ずっと症状が
続いていなくても可

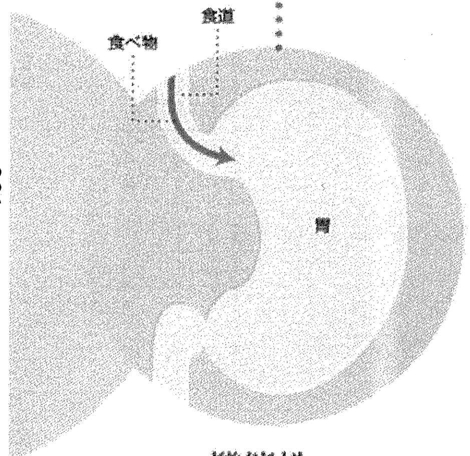
## ❸胃カメラなど胃の検査をしても「異常なし」といわれた

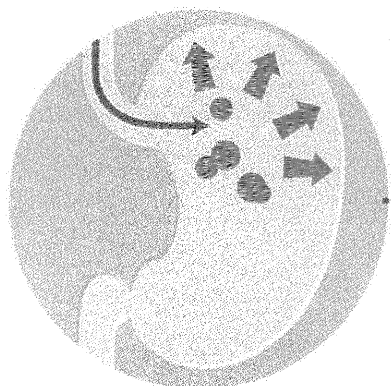
**機能性ディスペプシアの可能性が高いのは、❶〜❸すべてにあてはまる方です。**

# どうして起きる？
# 機能性ディスペプシア

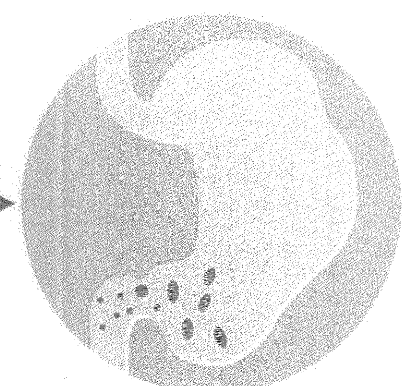
機能性ディスペプシアの原因は……
●ストレス
●胃がうまく動かなくなる（胃の運動機能障害）
●胃・十二指腸の知覚過敏
……などが考えられます

●健康な胃では…
口から入った食べ物は、
食道を通って胃に入ります



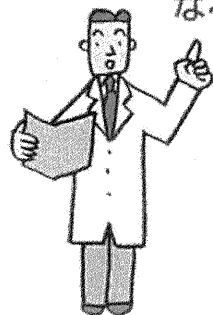


胃の上部がふくらみます

胃が波打つように動き（蠕動運動）、
食べ物と胃液がまざり、消化します


消化した食べ物を
十二指腸に送り出します



●機能性
ディスペプシアに
なると…



胃に食べ物が入っても、胃の上部が広がらず、すぐにおなかがいっぱいと感じてしまいます

●早期飽満感
●食欲不振

蠕動運動が弱くなって、十二指腸に食べ物を送りにくくなります　結果、胃の中に長く食べ物が残っている状態に

●胃もたれ　●胃が重い
●おなかの張り

胃液に含まれる胃酸などに対して、胃や十二指腸が敏感になって過剰に反応してしまいます

●みぞおちの痛み
●みぞおちの焼けるような感じ

# 機能性ディスペプシアと診断されたら……

※症状が改善しても、胃にやさしい生活を続けましょう

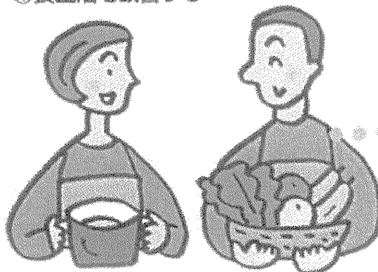
## 胃にやさしい生活を

①睡眠を十分とる

②ストレスをためない

③禁煙する

④食生活を改善する



### 症状が重いときには、薬物療法も……

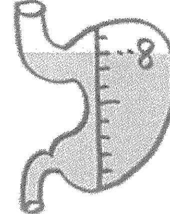
- ■胃酸の分泌を抑える薬
- ■胃腸の動きを活発にする薬
- ■漢方薬
- ■抗うつ薬（心理的な要因が関係しているとき）

※くわしくは担当の医師と相談してください

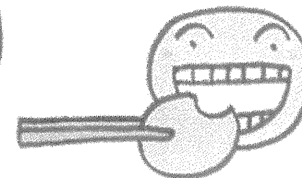
## 食生活の改善　これがポイント

1日3回、規則正しく

腹八分目に



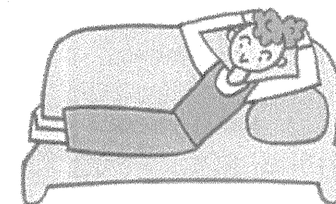

ゆっくり、
よく噛んで食べる



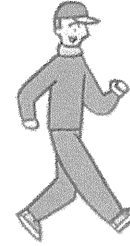
### こんな食品は控えめに

- 脂肪の多い肉や魚
- 甘いもの
- 塩辛いもの・刺激の強い香辛料

アルコールはほどほどに



食後は休憩を



監修

慶應義塾大学医学部内科学（消化器）准教授 …… 鈴木 秀和
国立国際医療研究センター国府台病院院長 …… 上村 直実
愛知医科大学消化器内科学教授 …… 春日井 邦夫
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慶應義塾大学医学部内科学（消化器）教授 …… 日比 紀文

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厚生労働科学研究費補助金（医療技術実用化総合研究事業）　研究代表者 鈴木秀和